Biochimica et Biophysica Acta, 1100 (1992) 9–14

BBABIO 43583

Spectral identification of the electrochromically active carotenoids of *Rhodobacter sphaeroides* in chromatophores and reconstituted liposomes

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(Received 3 October 1991)

Key words: Carotenoid bandshift; Pigment-protein complex; Light harvesting complex; Reconstitution; Chromatophore; (*Rb. sphaeroides*)

Reaction centers with both light harvesting complexes I and II (B875 and B800/850; i.e., $RCLH_1LH_{II}$ complexes) have been isolated from *Rhodobacter sphaeroides*. These complexes have been incorporated into liposomes made from lipids purified from *Escherichia coli*. The electrochromic bandshift of carotenoids, present in these reconstituted complexes, shows shifted minima and maxima with respect to a similar spectrum in chromatophores of *Rb. sphaeroides* in a potassium diffusion potential induced difference spectrum (see also Crielaard, W., Hellingwerf, K.J. and Konings, W.N. (1989) Biochim. Biophys. Acta 973, 205–211). The absorbance spectrum, at room temperature or at 77 K, of both membrane preparations did not, however, reveal differences in the carotenoid region. The long-wavelength carotenoid peak in both preparations is located at 513 nm (77 K). A small difference could be observed between the 77 K excitation spectra of the B850 fluorescence. Reconstituted complexes show a carotenoid peak at 513 nm, whereas in chromatophores this peak is located at 514.5 nm. When fluorescence was recorded at 805 nm, to detect B800 excitation, there was a marked difference between both preparations. In liposomes the long wavelength, B800-associated carotenoid peak is located at 512.5 nm, whereas in chromatophores this peak is located at 516 nm. These results explain the shifted minima and maxima in a potassium diffusion induced difference spectrum in proteoliposomes. The prediction of two carotenoid pools in chromatophores (De Grooth, B.G. and Amesz, J. (1977) Biochim. Biophys. Acta 462, 247–258) is confirmed, and the field sensitive carotenoids are identified as the pool that is associated with the B800 band (Kramer, H.J.M., Van Grondelle, R., Hunter, C.N., Westerhuis, W.H.J. and Amesz, J. (1984) Biochim. Biophys. Acta 765, 156–165).

Introduction

The electrochromic response of the carotenoid molecules associated with the antenna complexes of *Rhodospirillaceae* has been widely used to determine membrane potential values across membranes of photosynthetic bacteria [1–3]. The carotenoid absorption change has the advantage of a rapid response over other methods for recording membrane potentials (see, for example, Ref. 4). This method therefore allows measurement of actual *changes* of the membrane potential, which is not possible with the widely used distribution procedure of lipophilic ions since the diffusion of these probes across the membrane is not sufficiently fast (see, for example, Refs. 3,5). Another advantage of the carotenoid bandshift as membrane potential indicator is the linear relationship between the membrane potential and the absorption change [1], which makes it possible to read directly the membrane potential (change) in vivo.

Recently, it became also possible to use carotenoid absorbance changes to record membrane potentials in membranes in which carotenoids are not endogenously present. After the reconstitution of pigment-protein complexes from *Rhodobacter sphaeroides*, containing the light-harvesting-II (LH_{II}) complex of the antenna system (to which the electric-field-sensing carotenoid is bound, see Refs. 6–8) into liposomes, potential-induced absorbance changes of the associated carotenoids

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Abbreviations: RC, reaction center; $LH_{I/II}$, light-harvesting complex I (II); BChl, bacteriochlorophyll; octyl glucoside, n-octyl β-D-glucopyranoside.

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were observed [9]. The importance of this development is obvious: the ability to measure membrane potential changes on a rapid time scale can now be exploited in non-photosynthetic membranes. By the membrane fusion technique the bacterial reaction center/antenna complex can be introduced in membranes of fermentative bacteria [10]. The relation between the membrane potential and the carotenoid absorbance change in the reconstituted liposomal system is linear, as in native photosynthetic membranes [1]. The potential-induced difference spectrum of these reconstituted carotenoids is, however, shifted approx. 4–5 nm to shorter wavelengths with respect to the difference spectrum of *Rb. sphaeroides* chromatophores [9]. Since (at room temperature) no shifts can be observed in an overall absorbance spectrum of the total pool of reconstituted carotenoids (as is the case for lithium-dodecylsulphate treated pigment-protein complexes from *Rhodopseudomonas acidophila* [11]) it was suggested that only the field-sensitive (presumably B800) carotenoids display an altered spectrum [9]. The actual change in position of the absorption peak of the B800 carotenoids might be obscured by the (non-shifted) B850 and B875 carotenoids (for explanation of the carotenoid nomenclature see, for example, Ref. 12). There are strong indications that two functionally different types of carotenoid exist within the LH_{II} complex [12], one type associated with the B800, and one associated with the B850. In chromatophores the magnitude of the electrochromic signal is proportional to the amount of LH_{II} [8]. Since the B850 carotenoid pool is believed to be structurally similar to the carotenoids in the B875 complex, which are not field-sensitive, it has been suggested that the B800 carotenoids are the field-sensitive pigments.

Here we use and test these assertions in an attempt to explain the observed difference between the band-shift spectra of chromatophores and reconstituted $RCLH_{I}LH_{II}$ complexes [9]. Since the field-sensitive carotenoids are supposed to be the only carotenoids that transfer energy to B800, the excitation spectrum of the emission of the B800 should contain only this pool of carotenoids. In order to obtain a pure excitation spectrum of the B800 emission we performed fluorescence spectroscopy at low temperature (77 K), to obtain B800 emission which is well separated from the B850 and B875 emission (also, possible contamination of the excitation spectrum by means of B850 to B800 back-transfer is thus prevented).

Materials and Methods

Growth of Rb. sphaeroides and preparation of chromatophores

Rb. sphaeroides, strain 2.4.1, was grown anaerobically at low light intensity (equidistant from two 60 W tungsten lamps placed 50 cm apart) in 1 litre bottles in the medium described by Sistrom [13] at 30°C. Cells were harvested at an A_{660} of approx. 3, washed twice in 50 mM potassium phosphate (pH 7.6), 50 mM KCl, 8 mM $MgCl_2$, 10% sucrose and resuspended in this buffer.

Chromatophores were prepared from these cells by two successive passages through a French pressure cell at 18000 psi, 0°C. Debris was removed from the preparation by low-spin centrifugation (30 min, $20000 \times g$, 0°C). Chromatophores were collected by ultracentrifugation (1 h, $200000 \times g$, 4°C) and resuspended in 50 mM potassium phosphate (pH 7.6), 50 mM KCl, 8 mM $MgCl_2$, 10% sucrose, to 1–1.5 mM bacteriochlorophyll (BChl). The chromatophores were kept on ice until further use.

Isolation of pigment-protein complexes

Pigment-protein complexes were isolated from chromatophores of *Rb. sphaeroides* according to a modified procedure from Varga and Staehelin [14] as described by Molenaar et al. [15] for *Rhodopseudomonas palustris*, but using cholate instead of n-octyl β-D-glucopyranoside (octyl glucoside). The $RCLH_{I}LH_{II}$-complexes were extracted from the membranes with 1.5% cholate in 50 mM potassium phosphate (pH 7.6), 50 mM KCl, 8 mM $MgCl_2$, 10% sucrose plus 20 mM K^+-EDTA for 1 h at 0°C. The mixture was vortexed every 5 min during solubilization. Non-solubilized material was removed by centrifugation (2 min in an Eppendorf centrifuge). The supernatant (0.3 ml) was layered on a 9 ml linear sucrose gradient with 10–50% sucrose (w/v) in 50 mM potassium phosphate (pH 7.6), 50 mM KCl and 1.5% cholate. Gradients were centrifuged in a Beckman SW 41 Ti rotor (19 h, 35000 rpm, 6°C). Pigmented bands were recovered from the gradient, analyzed spectrophotometrically (see Ref. 15) and kept on ice until further use.

Incorporation of pigment-protein complexes into liposomes

Acetone-washed *Escherichia coli* phospholipids (mainly phosphatidylethanolamine, Sigma, St. Louis, MO, USA), dispersed in 50 mM potassium phosphate (pH 7.6), 50 mM KCl and 1% cholate, was sonicated to clarity under a constant stream of nitrogen gas at 0°C, using a probe type sonicator (MSE Scientific Instruments, West Sussex, UK) at an output of 2 μm. After the addition of pigment protein complexes (at a BChl/lipid ratio of 40 nmol/mg) the mixture was dialysed at 4°C for 20 h against a 1000-fold volume of 50 mM potassium phosphate (pH 7.6), 50 mM KCl (three changes). After dialysis the liposomes were stored in 1 ml aliquots in liquid nitrogen. Before use, the liposomes were thawed slowly at room temperature and sonicated twice for 3 s at 0°C with the probe-type

sonicator, at an output of 2 μm. The liposomes were concentrated in 50 mM potassium phosphate (pH 7.6), 50 mM KCl by ultracentrifugation (1 h, 200000 × g, 4°C) and again sonicated two times for 3 s (0°C, 2 μm) to remove aggregates.

Potassium diffusion potentials

Potassium diffusion potentials, inside negative, were induced in chromatophores and a concentrated suspension of liposomes, by diluting the samples in 50 mM sodium phosphate (pH 7.6), 50 mM NaCl (supplemented with 10% sucrose and 8 mM $MgCl_2$ in the case of chromatophores) and adding 100 nM valinomycin.

Room-temperature absorbance changes

Absorbance difference (bandshift) spectra were recorded and analyzed using a single-beam Philips PU8700 Series UV/VIS spectrophotometer. Spectra were plotted using the medium smoothing mode. All experiments were performed at 20°C in a 3 ml quartz cuvette with continuous stirring.

Low-temperature absorbance and fluorescence-excitation spectra

Low-temperature samples were prepared in 50% glycerol, 20 mM Tris-HCl (pH 8.0), in 1 cm acrylic fluorescence cuvettes. Samples were cooled using an Oxford DN1704 liquid Nitrogen cryostat (equipped with an ITC 4 temperature controller).

Low-temperature absorption spectra were recorded on a Cary 219 spectrophotometer. Fluorescence measurements were performed on a home-built fluorimeter [16]. Fluorescence was detected at right-angles, through a double f3.5 monochromator, using an EMI 9658 photomultiplier (cooled to −30°C to reduce dark current).

Absorption and excitation spectra were analyzed on a Sun 4/280 minicomputer using a home-developed program. Gaussian fits of spectra were deduced using the Marquardt-Levenberg algorithm.

Analytical procedures

Bacteriochlorophyll (BChl) was estimated at 772 nm in acetone/methanol extracts according to Clayton [17].

Results

Absorbance changes in chromatophores and reconstituted liposomes, induced by an imposed membrane potential

In intact bacterial cells and chromatophores the electrochromic carotenoid absorbance changes show a characteristic spectrum, which can be explained by a shift (to higher or lower wavelength, dependent on the direction of the externally applied field) of the absorbance bands of a red-shifted fraction of the carotenoids [18,19]. Such a characteristic spectrum could also be observed after isolation and reconstitution of $RCLH_1LH_{II}$ complexes into liposomes (Fig. 1). For comparison, also the spectral changes in the same region were recorded for *Rb. sphaeroides* chromatophores from which the pigment-protein complexes were isolated. Fig. 1 shows the result of these analyses. Chromatophores displayed a diffusion-potential induced difference spectrum with maxima at 521, 488 and 458 nm and minima at 506, 472 and 442 nm (Fig. 1B). Liposomes containing $RCLH_1LH_{II}$ complexes showed a similarly shaped difference spectrum (Fig. 1A), but with different maxima (499 nm and 467 nm) and minima (517 nm, 482 nm and 451 nm).

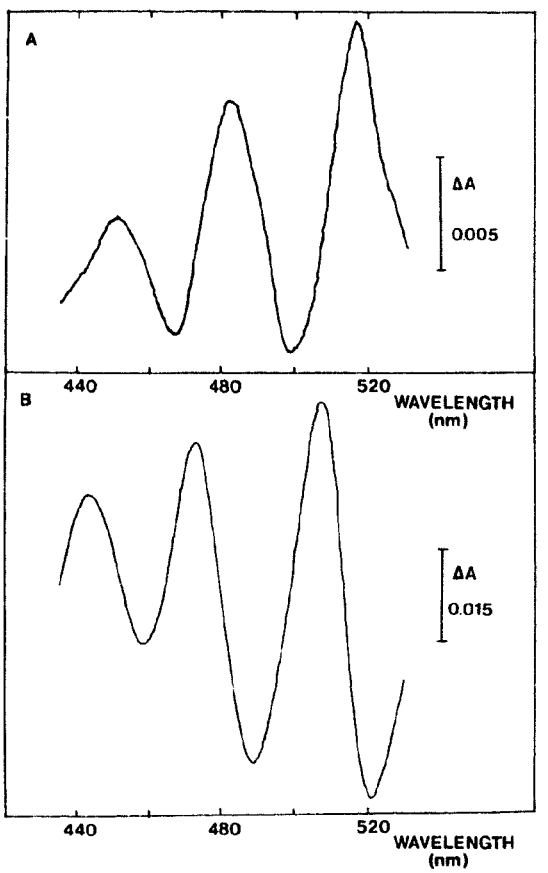

Fig. 1. Spectra of the carotenoid absorbance change induced by a K^+ diffusion potential in chromatophores of *Rb. sphaeroides* and liposomes containing $RCLH_1LH_{II}$-complexes. K^+-loaded chromatophores and liposomes (reconstituted at 40 nmol BChl/mg lipid) were diluted 50-fold into 2 ml 50 mM sodium phosphate (pH 7.6) 50 mM NaCl (supplemented with 10% sucrose and 8 mM $MgSO_4$ in the case of chromatophores). The figure shows the difference between spectra before and after the addition of 100 nM valinomycin. (A) $RCLH_1LH_{II}$-liposomes, final BChl concentration, 8.8 μM; (B) Chromatophores, final BChl concentration, 36 μM.

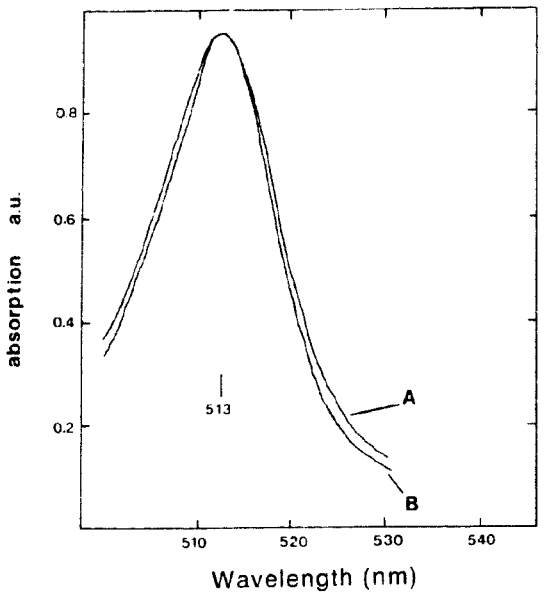

Fig. 2. Low-temperature absorbance spectra of (A) *Rb. sphaeroides* chromatophores and (B) $RCLH_1LH_{II}$-liposomes. Both samples were prepared as described in Materials and Methods. Liposomes were reconstituted at 40 nmol BChl/mg lipid. Both spectra were recorded at 77 K (a.u., arbitrary units).

Absorbance spectra

A small blue-shift (4 nm) has recently been observed in the absorption spectrum of carotenoids in the LH_{II} complex from *Rps. acidophila*, upon incubation with a low concentration of an ionic detergent (lithium dodecyl sulphate) [11]. We therefore investigated whether a similar blue-shift between (part of the) carotenoids in chromatophores and in reconstituted $RCLH_1LH_{II}$-liposomes was also observable in our preparations. The peak positions of the carotenoids in the absorbance spectrum of $RCLH_1LH_{II}$-liposomes, at room temperature, do not differ measurably from those of *Rb. sphaeroides* chromatophores (data not shown). This is also the case when the absorbance spectra are recorded at 77 K (Fig. 2). Both membrane preparations display, for the long-wavelength carotenoid peak, an absorbance maximum at 513 nm. However, even at 77 K the inferred shift of the field-sensitive B800 carotenoids may be obscured by the other carotenoids in the sample (RC, B875 and B850), which are present in both preparations abundantly and do not necessarily show a shift identical to that of the field-sensitive carotenoids.

Fluorescence-excitation spectra

To identify the absorbance peaks of the B800- and B850-associated carotenoids, fluorescence-excitation spectra were recorded at 77 K. Figs. 3 and 4 display low-temperature excitation spectra of respectively

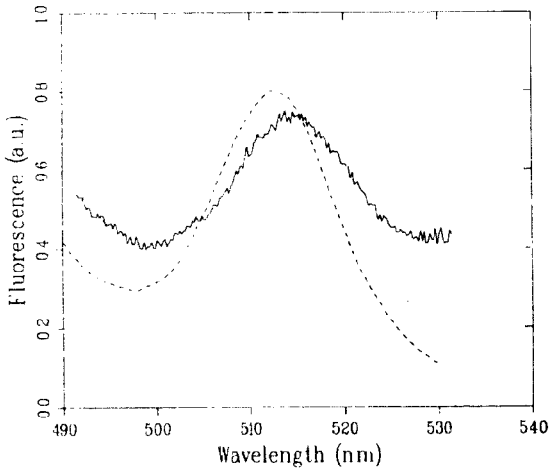

Fig. 3. Fluorescence-excitation spectra of *Rb. sphaeroides* chromatophores. Dotted line: Emission detected at 860 nm. Solid line: Emission detected at 805 nm. Dashed line: Emission detected at 910 nm. The sample was prepared as described in materials and methods. All three spectra were recorded at 77 K (a.u., arbitrary units).

chromatophores and $RCLH_1LH_{II}$-liposomes, when the fluorescence emission was detected at 850/860, 805 and 910 nm. Fig. 3 shows the excitation spectra of the redmost absorption band of the carotenoid region of

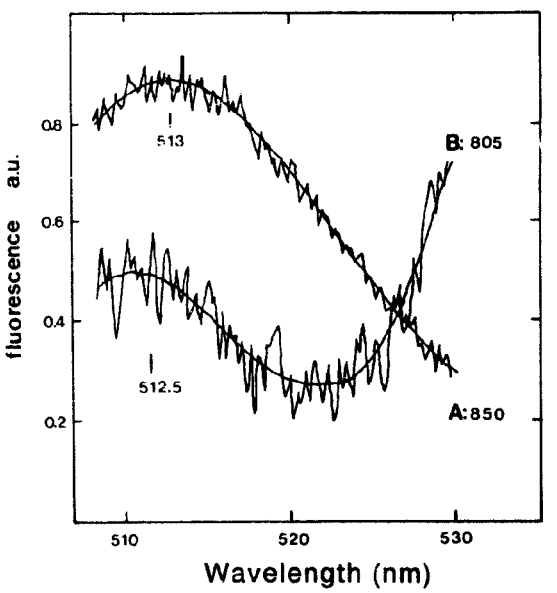

Fig. 4. Fluorescence-excitation spectra of $RCLH_1LH_{II}$-liposomes. (A) Emission detected at 850 nm. (B) Emission detected at 805 nm. The sample were prepared as described in Materials and Methods. Liposomes were reconstituted at 40 nmol BChl/mg lipid. Both spectra were recorded at 77 K. The solid lines represent Gaussian fits (a.u., arbitrary units).

chromatophores of *Rb. sphaeroides* (corrected for the lamp spectrum). The excitation peak detected at 910 nm (513 nm) illustrates LH_I emission, which represents the sum of the contributions from all carotenoids, including the B875 carotenoids, and appears at an identical position as the absorption peak. From Fig. 3 it is clear that the LH_{II} carotenoids contribute mainly to the red side of the absorption band. Also, there is a slight difference between the B800 and B850 excitation spectrum. The red side of the B800 excitation spectrum is contaminated by the excitation spectrum of some uncoupled pigments. This contamination, which was variable in different preparations, had no significant effect on the position of the maximum of the B800 excitation spectrum.

The difference between the B800 and B850 excitation spectra (Fig. 3) is only small (514.5 vs. 516). This is due partly to the fact that the B800 spectrum reflects a single component, while the B850 excitation spectrum contains contributions from both the B800 and the B850 carotenoids, due to very efficient energy transfer from B800 to B850 [20,21]. This means that the true position of the B850 carotenoid band is slightly more to the blue, and that there is a significant difference between the two carotenoid pools. Such a difference was not observed in similar excitation spectra of $RCLH_ILH_{II}$-liposomes (Fig. 4). Both the excitation spectrum of the B850 carotenoids (513 nm) and the B800 carotenoids (512.5 nm) are nearly identical to the absorption spectrum. This indicates that the absorbance peak of the B800 carotenoids in liposomes is indeed blue-shifted several nanometres with respect to the B800 carotenoids in chromatophores.

Discussion

Spectral differences between chromatophores and reconstituted liposomes

The most consistent interpretation of the bandshift spectra (Fig. 1) assumes that solubilisation and/or reconstitution of the pigmented proteins causes a slight blue-shift (approx. 4 to 6 nm) of the field-sensitive carotenoids (i.e., those associated with LH_{II}; see Refs. 6–8). When this blue-shift is taken into account, it is clear that the carotenoids in chromatophores and in $RCLH_ILH_{II}$ complexes give a mirror-response when exposed to a membrane potential. This is caused by the opposite orientation of the carotenoids associated with LH_{II} in these two samples. The results described in Fig. 1 make it unlikely that the complexes have been reconstituted in a random orientation. In that case, a doubling of the number of minima and maxima in the potential-induced difference spectrum would be expected. Indeed, it was found that in $RCLH_ILH_{II}$ complexes, reconstituted in *E. coli* lipids, more than 95% of the cytochrome *c* binding sites were exposed to the external aqueous phase (Crielaard, W. and Hellingwerf, K.J., unpublished observations). This is the same direction and degree of net orientation as observed for $RCLH_ILH_{II}$ complexes from *Rhodopseudomonas palustris* after reconstitution in *E. coli* phospholipids [15] and contrasts their orientation in chromatophores.

No significant differences can be observed between the absorbance spectrum of the two preparations (Fig. 2), indicating that the carotenoids which show an altered spectrum after reconstitution represent only a small part of the carotenoids present in the preparations. The major part of the carotenoids (e.g., those associated with the RC, B850 and B875) is therefore not measurably affected by reconstitution. Indeed when emission is detected at 910 nm in chromatophores (Fig. 3; representing LH_I emission and therefore both LH_I and LH_{II} excitation) an excitation peak is recorded which shows the same maximum as the absorbance spectrum (513 nm).

It is clear that chromatophores contain a heterogeneous pool of carotenoids, showing different excitation spectra. After reconstitution only a part of this pool (the B800 carotenoids) is damaged and shows an altered spectrum.

The observed difference between the B800 excitation spectra of chromatophores and liposomes is sufficient to explain the shifted bandshift spectra of the liposomes. This conclusion depends on the assertion that the B800 carotenoid is the field-sensitive pigment. However, the correlation between the bandshift spectra and the B800 excitation spectra is a very strong indication that this is actually the case.

Electrochromic implications

The shift in the absorption peak of the B800 carotenoid after reconstitution is probably caused by a slight change in the conformation of the LH_{II} protein. This change of conformation may cause a change in the polarizing permanent field near the carotenoid. According to the model of Kakitani et al. [22], this field (presumably resulting from charged amino acids) is responsible for both the location of the absorption peak of the B800 carotenoid and for the linearity of the electrochromic response. This proposed effect of the protein environment on the field sensitivity was recently confirmed by Stark effect measurements performed on the isolated B800–850 complex [23] (note that also in these *isolated* complexes, as in reconstituted liposomes, no difference was detected between the absorption peaks of the different carotenoids, suggesting that the B800 carotenoid in these preparations had also been altered). In the proteoliposomes the electrochromic response is still linearly dependent on the applied field [9], indicating just a small change of the permanent field.

The existence of a 'second pool' of carotenoids (absorbing several nanometres to longer wavelengths) was already suggested by De Grooth and Amesz [19]. Analysis of the overall carotenoid spectrum in *Rb. sphaeroides* chromatophores into Gaussian components, by these investigators, revealed two different carotenoid pools. A first pool, containing about 35% of the total carotenoid, which responds to potential changes and a second pool with absorbance maxima at 5–9 nm shorter wavelengths, which do not show absorbance changes [19]. The studies presented here confirm this hypothesis. We are currently trying to gain more insight into the exact nature of the two pools using site-directed mutagenesis on the protein subunits of the LH_{II} complex.

The fact that a shift of approx. 3.5 nm to shorter wavelengths does not abolish the field sensitivity of the B800 carotenoid, confirms the model predicted by Kakitani et al [22]. These authors suggested that charged residues of the antenna proteins were responsible for the spectral red-shift of both the responding and the non-responding carotenoid pools. For the linear electrochromic response it is, however, also essential that the charge is asymmetrically distributed around the carotenoid molecule. This means that the location of the absorbance peak is not essential for electrochromic response, but rather the presence of a polarizing electrical field. This field appears to be still large enough in the reconstituted LH_{II} (see also Ref. 9), since field-sensitivity is preserved in these reconstituted complexes.

Therefore, the data obtained with the liposomes can be consistently explained within the electrochromic theory [22]. This may allow the use of carotenoid absorbance changes as a membrane-potential-registration method in reconstituted and fused membranes.

Acknowledgements

This study was supported by the Netherlands Organization for the Advancement of Pure Research (NWO), via the Netherlands Foundation for Chemical Research (SON) and the Netherlands Foundation for Biophysics (SvB).

References

1 Jackson, J.B. and Crofts, A.R. (1969) FEBS Lett. 4, 185–189.
2 Clark, A.J. and Jackson, J.B. (1981) Biochem. J. 200, 389–397.
3 Crielaard, W., Cotton, N.P.J., Jackson, J.B., Hellingwerf, K.J. and Konings, W.N. (1988) Biochim. Biophys. Acta 932, 17–25.
4 Jackson, J.B. and Crofts, A.R. (1971) Eur. J. Biochem. 18, 120–130.
5 Demura, M., Kamo, N. Kobatake, Y. (1985) Biochim. Biophys. Acta 812, 377–386.
6 Matsuura, K., Ishikawa, T. and Nishimura, M. (1980) Biochim. Biophys. Acta 590, 339–344.
7 Webster, G.D., Cogdell, R.J. and Lindsay, J.G. (1980) Biochim. Biophys. Acta 591, 321–330.
8 Holmes, N.C., Hunter, C.N., Niederman, R.A. and Crofts, A.R. (1980) FEBS Lett. 115, 43–48.
9 Crielaard, W., Hellingwerf, K.J. and Konings, W.N. (1989) Biochim. Biophys. Acta. 973, 205–211.
10 Crielaard, W., Driessen, A.J.M., Molenaar, D., Hellingwerf, K.J. and Konings, W.N. (1988) J. Bacteriol. 170, 1820–1824.
11 Robert, B. and Frank, H.A. (1988) Biochim. Biophys. Acta 934, 401–405
12 Kramer, H.J.M., van Grondelle, R., Hunter, C.N., Westerhuis, W.H.J. and Amesz, J. (1984) Biochim. Biophys. Acta 765, 156–165.
13 Sistrom, W.R. (1960) J. Gen. Microbiol. 22, 778–785.
14 Varga, A.R. and Stachelin, L.A. (1985) J. Bacteriol. 161, 921–927.
15 Molenaar, D., Crielaard, W. and Hellingwerf, K.J. (1988) Biochemistry 27, 2014–2023.
16 Visschers, R.W., Chang, M.C., Van Mourik, F., Parkes-Loach, P.S., Heller, B.A., Loach, P.A. and Van Grondelle, R. (1991) Biochemistry 30, 5734–5742.
17 Clayton, R.K. (1960) Biochim. Biophys. Acta 75, 312–323.
18 De Grooth, B.G. and Amesz, J. (1977) Biochim. Biophys. Acta 462, 237–246.
19 De Grooth, B.G. and Amesz, J. (1977) Biochim. Biophys. Acta 462, 247–258.
20 Van Grondelle, R., Bergström, H., Sundström, V. and Gillbro, T. (1987) Biochim. Biophys. Acta 894, 313–326.
21 Bergström, H., Sundström, V., Van Grondelle, R., Gillbro, T. and Gogdell, R.J. (1988) Biochim. Biophys. Acta 936, 90–98.
22 Kakitani, T., Honig, B. and Crofts, A.R. (1982) Biophys. J. 39, 57–63.
23 Gottfried, D.S., Steffen, M.A. and Boxer, S.G. (1991) Science 251, 663–665.